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Involvement of B Lymphocytes in the Growth Inhibition of Human Pulmonary Melanoma Metastases in Athymic *nu/nu* Mice by an Antibody-Lymphotoxin Fusion Protein¹

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Abstract

Antibody-cytokine fusion proteins can target biologically active cytokines to various tumor sites, achieving local concentrations sufficient to induce host immune responses leading to tumor elimination. Here, we demonstrate the therapeutic efficacy of a tumor-specific antibody-lymphotoxin fusion protein (ch225-LT) on xenografted pulmonary metastases of human melanoma. *In vitro* studies indicated a direct cytotoxic effect of such constructs on melanoma cells via the induction of apoptosis, as demonstrated by cell cycle analysis and DNA fragmentation. However, ch225-LT lacked any therapeutic effect in immune deficient C.B17 *scid*/*beige* and *scid/scid* mice, indicating the insufficiency of this direct mechanism *in vivo*. In contrast, in athymic *nu/nu* mice, ch225-LT completely inhibited outgrowth of the xenografted tumor. This therapeutic effect was accompanied by infiltrations of CD45+, Mac-1+, and asialo-GM1+ cells into the tumor; B220+ cells were present in the surrounding tissue and the periphery of the tumor. The functional role of asialo-GM1+ cells was confirmed by *in vivo* depletion studies. Our data indicate that an antibody-lymphotoxin fusion protein effectively inhibits the growth of disseminated melanoma metastases by mechanisms that function in the absence of mature T cells, but require B, NK, and other asialo-GM1+ cells.

Introduction

LT³ is closely related to TNF. LT is a *M*_r 25,000 glycoprotein produced by mitogen-activated T, B, and LAK cells (1, 2). LT and TNF share two common receptors, p80 and p60, and have many similar activities, including cytotoxic effects on some neoplastic cells *in vitro* (1, 3). TNF secreted by tumor-specific TIL cell clones isolated from the primary and secondary melanoma of patients was reported to have a direct cytotoxic effect against the relevant tumor (4). Human LT was shown to enhance the susceptibility of preneoplastic and neoplastic cells to NK cell-mediated cytotoxicity (5). It exerts at least equal antitumor activity in comparison to TNF and is less toxic in mice (6). Furthermore, only LT has a stimulatory effect on the growth of prestimulated B cells (7) and acts as an autocrine growth factor for EBV-infected cells (8). These characteristics made TNF and LT interesting candidates for tumor therapy. However, systemic application of effective doses of these cytokines has been prohibited by severe toxicities of such treatments. In clinical application, high doses of TNF could only be used regionally in the form of hyperthermic isolated limb perfusion, which proved to be an effective treatment of

metastatic melanoma confined to one extremity. However, TNF leakage occurred occasionally, which caused major systemic effects, consisting of cardiovascular, respiratory, and hematological disturbances (9).

The *ex vivo* transfection of tumor cells with genes leading to the expression of various cytokines offers a new approach for localized immunotherapy (10, 11). The induction of inflammatory responses by such cytokine-producing tumor cells led to their rejection upon inoculation into syngeneic mice and, in some cases, produced systemic immune responses against challenges with wild-type parental tumor cells. T cells were shown to play a key role in all such experiments in which systemic immunity was analyzed. However, Qin and Blankenstein (12) recently found evidence for B lymphocyte involvement in antitumor responses of syngeneic mice inoculated with murine plasmacytoma cells transfected with the human LT gene. This distinguishes LT from other cytokines analyzed in similar experiments. These investigators concluded that the LT-producing plasmacytoma cells are rejected by a complex immunological mechanism involving T cells, as well as B and other cells.

We previously described a novel cytokine-based immunotherapeutic approach for cancer treatment that is characterized by a simple *modus operandi* and effective local concentrations of cytokines in the tumor microenvironment. We achieved this goal by constructing fusion proteins consisting of tumor-specific mAbs and cytokines, thus utilizing the unique targeting ability of antibodies to direct cytokines to tumor sites (13-15). Here, we demonstrate that an antibody-LT fusion protein can effectively inhibit growth of disseminated pulmonary metastasis of human melanoma in athymic *nu/nu* mice. Because this therapy is not effective in either nude mice depleted of asialo-GM1+ cells, C.B17 *scid/scid* mice lacking in B and T cells, or in C.B17 *scid/beige* mice that additionally are deficient in NK cells, the antitumor effects achieved by the ch225-LT fusion protein involve a mechanism that functions in the absence of T cells, but requires B, NK, and other asialo-GM1+ cells.

Materials and Methods

Cell Lines and Reagents. The human melanoma cell line M24met, expressing the EGF receptor, has been described previously (16). This cell line was maintained as a monolayer in RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine and was passaged as necessary. The mouse/human chimeric antibody directed against the human EGF receptor (ch225) was constructed by joining the cDNA for the variable region of the murine antibody with the constant region of the γ 1 heavy chain and the κ light chain, as reported previously (17). The antibody-LT fusion protein (ch225-LT) was constructed by fusion of the LT cDNA to the γ 1 H chain constant region at the end of the third (CH3) domain, as described for a LT fusion protein with antiganglioside GD2 mAb ch14.18 (18). The fused genes were inserted into the vector pHL2 encoding the ch225 V regions and the dihydrofolate reductase gene. The resulting expression plasmid was introduced into Sp20-Ag14 cells by proto-

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³ The abbreviations used are: LT, lymphotoxin; TNF, tumor necrosis factor; mAb, monoclonal antibody.

plast fusion and selected in DMEM supplemented with 10% fetal bovine serum and 100 nM methotrexate. The fusion protein was purified over a protein A-Sepharose affinity column, as described previously (18). All other antibodies used are commercially available and have been described by their respective manufacturers (PharMingen, San Diego, CA, and WAKO Bioproducts, Richmond, WA).

Animals. C.B17 *scid/scid* and athymic *nu/nu* mice were obtained from Taconic Farms at the age of 6 weeks. C.B17 *scid/beige* mice were obtained from The Jackson Laboratory at the age of 4–6 weeks. These animals were housed under specific pathogen-free conditions and all experiments were performed according to NIH guidelines for care and use of laboratory animals.

In Vivo Depletion with mAb. Rabbit anti-asialo-GM1 antiserum (WAKO Bioproducts) was used for *in vivo* depletion experiments. Protocols leading to maximum depletion, as determined by indirect immunofluorescence staining and cytofluorometric analysis, consisted of injections of 100 μ l of antiserum on days -3, 0, 7, 14, and 21.

Tumor Models. Experimental lung metastases were induced by injection of single cell suspensions of 1×10^6 M24met cells into the lateral tail vein. To prevent pulmonary embolism caused by injection of tumor cells, mice were anesthetized by halothane inhalation, and tumor cells were suspended in 500 μ l PBS containing 0.1% BSA and administered i.v. over a period of 60 s. At day 35 after tumor cell injection, grossly visible metastases were present on the surface of the organ. Lungs were fixed in Bouin fixative and examined under a low-magnification microscope for tumor foci on their surface. Sections from the lungs were stained with H&E and examined histologically. Treatment of animals was started 24 h after induction of metastases and consisted of i.v. injection of either 0.5 μ g LT, 4 μ g ch225-LT, 16 μ g ch225-LT, or PBS for 7 days. Animals were sacrificed 28 days after tumor cell inoculation.

S.c. tumors were induced by s.c. injection of 2.5×10^6 M24met cells in RPMI 1640, which resulted in tumors of approximately 50 μ l volume within 14 days. Tumors and surrounding tissues were excised at indicated time points and snap frozen.

Flow Cytometry. Cells (1×10^6) were washed twice with PBS and incubated for 30 min with the primary mAb ch225 or the ch225-LT fusion protein. After two washes with HBSS containing 0.2% sodium azide, cells were stained for 30 min with a mouse antihuman IgG FITC conjugate. All incubations and washes were performed at 4°C. Cells were analyzed with a FACScan (Becton Dickinson, San Jose, CA) at 488 nm.

Cytotoxicity Assay. Cell lysis was measured in an 18-h ^{51}Cr -release assay. U-bottomed plates were used for cytotoxicity assays in conjunction with RPMI 1640 supplemented with 10% FCS. Assays were performed at various rLT and ch225-LT concentrations using 2.5×10^4 ^{51}Cr -labeled L-929 murine sarcoma cells/well. Plates were incubated for 18 h, then a 70- μ l aliquot was removed from each well, and the specific ^{51}Cr -release was measured. Results were expressed as:

% cytotoxicity =

$$\frac{\text{experimental release (cpm)} - \text{spontaneous release (cpm)}}{\text{maximum release (cpm)} - \text{spontaneous release (cpm)}} \times 100$$

where spontaneous release was assessed by incubating target cells in the absence of LT, and maximum release was determined in the presence of 1% Triton X-100.

Cell Cycle Analysis. Cell cycle analysis of M24met melanoma cells was performed by measuring nuclear DNA content after incubation for 24 h at 37°C with either mAb ch225, rhLT, or ch225-LT fusion protein. Briefly, M24met cells were fixed in ice-cold 70% ethanol overnight at 4°C and stained for 30 min at room temperature with propidium iodide (50 μ g/ml) containing 100 Kunitz units of RNase A. Cells were analyzed by flow cytometry in a fluorescence-activated cell sorter.

Isolation of Low-Molecular-Weight DNA. DNA fragmentation was measured by isolation of low-molecular-weight DNA from M24met cells after treatment with either mAb ch225, rhLT, or ch225-LT. Briefly, cells were lysed in 0.1 M Tris-HCl buffer (pH 7.4), 10 mM EDTA, and 0.2% Triton X-100. After centrifugation at 14,000 g, DNA was extracted from the supernatant with phenol/chloroform. After ethanol precipitation, the DNA was resuspended in Tris-EDTA buffer (pH 7.4) and incubated with 100 Kunitz units of RNase for 60 min at 37°C before electrophoretic analysis on a 2% agarose gel for 45 min at 80 mV.

Immunohistology. Frozen sections were fixed in cold acetone for 10 min followed by removal of endogenous peroxidase with 0.03% H_2O_2 and blocking of collagenous elements with 10% species-specific serum in 1% BSA/PBS. The antibodies were then overlaid onto serial sections at predetermined dilutions (usually 20 μ g/ml), and slides were incubated in a humid chamber for 30 min. With PBS washes between all steps, a biotinylated link antibody was applied for 10 min, followed by another 10 min exposure to either peroxidase or alkaline phosphatase linked to streptavidin. The antibodies used were rat IgG2b anti-CD45 (clone 30F11.1), rat IgG2a anti-CD45R/220 (clone RA3-6B2), and rat IgG2b anti-Mac-1 (clone M1/70), all obtained from PharMingen (San Diego, CA).

Results

Prior studies demonstrated that fusion of a synthetic sequence coding for human LT to the carboxyl end of the human *Cyl* gene of mouse/human chimeric antiganglioside GD2 mAb ch14.18 resulted in a fusion protein that retained both the antigen binding capacity of the mAb and the cytokine activity of LT (18). A LT fusion protein utilizing the antigen binding capacity of the mouse/human chimeric antibody ch225 directed against the EGF receptor (ch225-LT) was made by the same procedure. Flow cytometry analysis of human melanoma cells M24met indicated that the ch225-LT fusion protein recognized EGF receptors expressed on this cell line equally well as its parent mAb ch225 (Fig. 1A). The functional activity of ch225-LT was demonstrated by its cytotoxic effect against murine L-929 fibrosarcoma cells. A comparison of the cytotoxic effect exerted by ch225-LT and rhLT against L-929 cells demonstrated that the fusion protein was approximately 10-fold less active on a weight basis but only 2-fold less active on a molar basis as measured by an 18-h ^{51}Cr -release assay (Fig. 1B).

Because members of the TNF "family" were reported to mediate tumor cell death (16, 17), we determined the direct cytotoxic effect of the ch225-LT fusion protein on M24met melanoma cells. As demonstrated by cell cycle analysis, both rhLT and ch225-LT effectively induced apoptosis in these cells (Fig. 2, A-C). This finding was confirmed by detection of DNA fragmentation after incubation with both rhLT and ch225-LT (Fig. 2D). The induction of apoptosis in tumor cells *in vivo* may provide an effective mechanism to control tumor growth without relying on the host immune system. To test this hypothesis, we used a number of inbred mouse strains displaying different immunodeficiencies for injection with human melanoma cells. In C.B17 *scid/beige* mice that are deficient in B, T, and NK cells, we found that neither rhLT nor the ch225-LT fusion protein had any inhibitory effect on the growth of pulmonary melanoma metastases (data not shown). Identical results were obtained when the same experimental protocol was used in C.B17 *scid/scid* mice that are deficient in B and T but not NK cells (data not shown).

Because neither rhLT nor the ch225-LT fusion protein induced antitumor effectors in C.B17 *scid/scid* or *scid/beige* mice in the absence of B, T, and NK cells, we addressed the question of whether B cells, in the absence of T cells could mediate antitumor effects after stimulation with rhLT or ch225-LT. We tested this hypothesis in athymic *nu/nu* mice that lack T but not B cells. The data of two separate experiments shown in Table I suggest that B cells are indeed involved in the eradication of pulmonary melanoma metastases induced by the ch225-LT fusion protein. It is evident from these experiments that ch225-LT was quite effective in this regard, whereas rhLT was ineffective at the dose level tested. The antitumor effect induced by ch225-LT is dose-dependent, as it is less pronounced at the 4- μ g than the 16- μ g dose level (Table I, Experiment I).

Because athymic *nu/nu* mice possess both B and NK cells, we addressed the question of whether B cells alone were involved in the antitumor effects induced by ch225-LT. To this end, we depleted

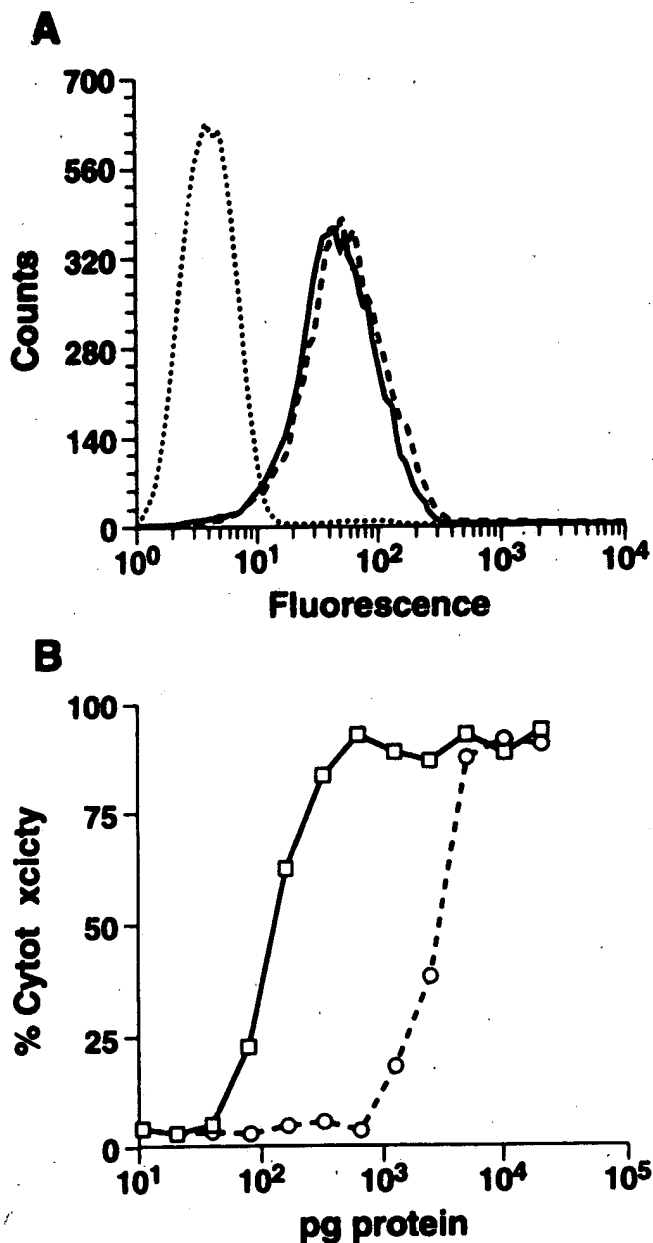


Fig. 1. Biological activities of the ch225-LT fusion protein. A, flow cytometry of 1×10^6 M24met cells incubated 30 min with PBS (.....), ch225 (-----), and ch225-LT fusion protein (—). B, cytotoxicity assay of 2.5×10^4 ^{51}Cr -labeled L-929 murine sarcoma cells with various concentrations of rhLT (\square) and ch225-LT fusion protein (\circ).

asialo-GM1+ cells *in vivo* with a polyclonal rabbit antibody and then tested the effect of ch225-LT against M24met pulmonary metastases in these animals. The data depicted in Table 2 indicate that the mechanism involved in the antitumor effect induced by ch225-LT is rather complex and requires not only B cells, but also a variety of asialo-GM1+ cells. Further information on the cellular mechanism involved was provided by immunohistochemical analysis of established s.c. tumors. We demonstrated previously that antibody-cytokine fusion proteins accumulate in s.c. tumors expressing the respective target antigen (11, 12). In this study, four animals each were treated daily over a period of 7 days by i.v. administered PBS (0.2 ml), rhLT (0.5 μg), or ch225-LT fusion protein (16 μg) starting 10 days after tumor cell inoculation. Biopsies were taken on days 4, 7, and 10 after the start of therapy. Inflammatory infiltrates were very obvious on day 7. Biopsies from tumors ($\sim 50 \mu\text{l}$ volume) of control animals

(PBS) and mice injected with rhLT revealed almost no infiltrates either within or at the periphery of the tumor, with only occasional CD45+ and asialo-GM1+ cells (data not shown). In contrast, tumors of mice treated with the ch225-LT fusion protein showed massive infiltration of CD45+ (Fig. 3A), Mac-1+ (Fig. 3B) and asialo-GM1+ cells (Fig. 3C). Furthermore, B220+ cells could be detected at the tumor site; however, these cells were restricted to the periphery of the tumor and the surrounding tissues (Fig. 3D).

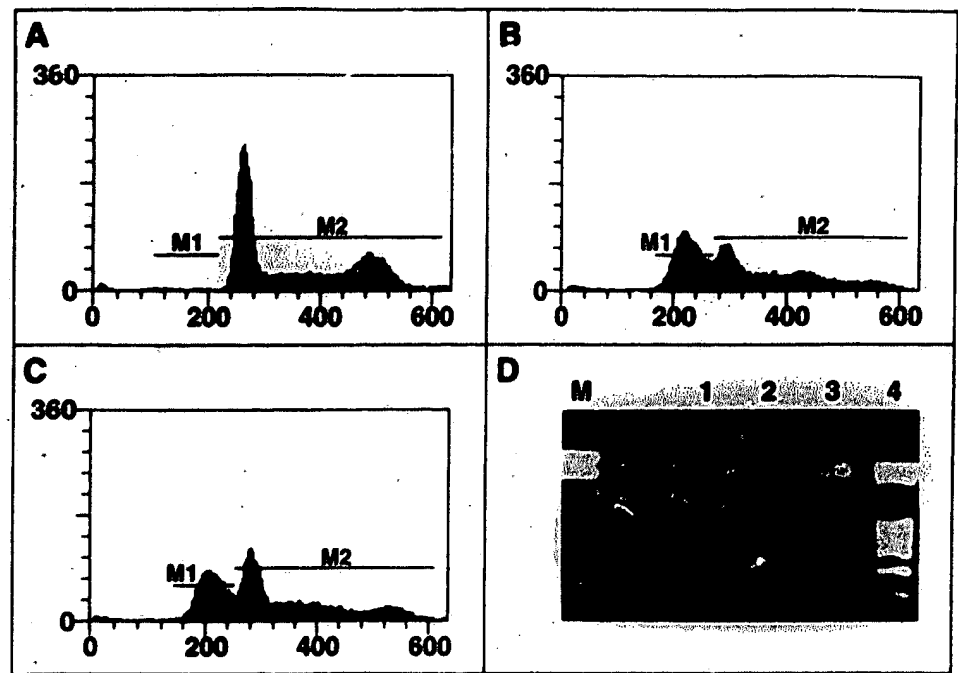
Discussion

We recently reported that antibody-cytokine fusion proteins can effectively target cytokines to tumor sites, achieving local concentrations sufficient to induce eradication of disseminated metastases (14, 15). We demonstrate here the therapeutic efficacy of an anti-EGF receptor antibody-LT fusion protein (ch225-LT) in inhibiting the growth of disseminated pulmonary metastases of human melanoma in athymic (*nu/nu*) mice. The antitumor effect of ch225-LT involves a complex immunological mechanism that functions in the absence of T cells but requires B cells and asialo-GM1+ cells, which are mainly NK cells. This conclusion is supported by the fact that growth of disseminated pulmonary melanoma metastases in C.B17 *scid/scid* or *scid/beige* mice could not be inhibited by the ch225-LT fusion protein. However, such metastases could be effectively eradicated by ch225-LT, but not rhLT, in athymic *nu/nu* mice. The requirement for NK cells in these animals was evident from depletion studies with anti-asialo-GM1 antibody before and during treatment with ch225-LT, resulting in complete abrogation of its antitumor effect. However, it should be noted that asialo-GM1 antiserum also reacts with non-NK cells, such as monocytes, bone marrow, spleen cells, and macrophages of nude mice. It is possible that the differences in efficacy of ch225-LT in C.B17 *scid/scid* and athymic *nu/nu* mice may also be influenced by differences in NK cell activities among these animals. In addition, antihuman B cell responses and production of IgM in athymic *nu/nu* mice bearing human M24met xenografts could also contribute to the therapeutic effect of ch225-LT, as such antibodies and NK cells might kill tumor cells by antibody-dependent cellular cytotoxicity.

Similar to our findings, Funahashi *et al.* (19) reported that the antitumor effect of LT was abrogated by anti-asialo-GM1 antibody in a syngeneic Meth A fibrosarcoma model. Our findings agree, in part, with those of a recent report by Qin and Blankenstein (12). These investigators used murine plasmacytoma J588L cells, transfected with a human LT expression plasmid and obtained an increased local concentration of this cytokine. They also found evidence for B lymphocyte involvement in athymic *nu/nu* mice, in which suppression of s.c. tumor growth was considerably more effective than in C.B17 *scid/scid* mice (12). It should be noted that a defect in B cell development of nude mice was suggested by Wortis *et al.* (20) based, in part, on a difference in electrophoretic mobility from that of normal B cells and the inability of added T cells to release nude mouse B cells after binding to macrophages; however, it is unlikely that this defect has an impact on our observations.

Immunological analysis confirmed the involvement of B and NK cells in the ch225-LT-mediated antitumor effect. Interestingly, asialo-GM1+ cells were found to be infiltrating the whole tumor, whereas B220+ cells were restricted to the tumor periphery. Qin and Blankenstein (12) demonstrated the presence of B cells throughout the entire tumor, provided the tumor cells were producing sufficient amounts of LT. These differences can be explained by the concentration gradients of LT in the tumor microenvironment created by these different forms of therapy, because antibody-targeted cytokine delivery produces higher concentrations in the periphery.

Fig. 2. Determination of cell death by apoptosis. Flow cytometry of 1×10^6 M24met cells incubated for 24 h with 100 μ g/ml ch225 (A), 0.15 μ g/ml rhLT (B), and 3 μ g/ml ch225-LT (C). D. DNA fragmentation in M24met melanoma cells: M, standard; Lane 1, M24met control; Lane 2, M24met + ch225 (100 μ g/ml); Lane 3, M24met + ch225-LT fusion protein (5 μ g/ml); Lane 4, M24met + rhLT (0.15 μ g/ml).



The effect of LT on B cells in our tumor model system is in accordance with previously reported *in vitro* data. LT was shown to be a growth factor for prestimulated human B cells (21) and was reported to act as an autocrine growth factor for human B cell lines (8) and for EBV-transformed B cells (10). Interactions between B and NK cells are suggested by a report that NK-mediated regulation of human B cells involves members of the TNF family of cytokines (22). A possible interaction between murine B cells and human LT is suggested by the finding that Hodgkin's disease-derived cell lines secreting large amounts of LT (23) grow as tumors in C.B17 *scid/scid*, but not in athymic *nu/nu* mice (24). In

addition, based on published data from several studies, LT can initiate growth stimulation of B cells (5) and the M_r 75,000 TNF/LT receptor molecule was shown to be functionally active when expressed on B cells (25). Activated B cells, in turn, are known to produce IL12, which induces the production of IFN- γ from NK cells, thus activating them and enhancing their cytotoxic activity against tumor target cells (26). Hypothetically, this is a mechanism that may be involved in the eradication of pulmonary melanoma metastases described in this study.

In summary, we have demonstrated that an antibody-LT fusion protein targets sufficient LT to disseminated pulmonary melanoma

Table 1. Therapeutic efficacy of ch225-LT on experimental lung metastases of human melanoma in athymic *nu/nu* mice

Treatment ^a	No. of foci ^b							
Experiment 1 ^c								
PBS	>500	>500	>500	>500	>500	>500	>500	>500
rhLT (0.5 μ g)	>500	>500	>500	>500	>500	>500	>500	>500
ch225-LT (4 μ g) ^d	12	14	26	100	>500	>500	>500	>500
ch225-LT (16 μ g) ^d	0	0	0	0	2	8		
Experiment 2 ^c								
PBS	>500	>500	>500	>500	>500	>500	>500	>500
rhLT (0.5 μ g)	>500	>500	>500	>500	>500	>500	>500	>500
ch225-LT (16 μ g)	0	0	0	0	0	2	8	21

^a Treatment was started 24 h after inoculation of tumor cells and consisted of i.v. injection of either PBS (0.1 ml), rhLT (0.5 μ g), or ch225-LT at either 4 μ g or 16 μ g daily over 7 consecutive days, as indicated. Animals were sacrificed 28 days after tumor cell inoculation.

^b All experimental groups were started with eight mice. Animals found dead before the planned date of sacrifice were not included in the evaluation.

^c Experimental lung metastases were induced by i.v. injection of 1×10^6 M24met melanoma cells into athymic *nu/nu* mice.

^d Differences in the number of metastatic foci between the groups of mice receiving either 4 μ g or 16 μ g ch225-LT daily for 7 days were statistically significant ($P \leq 0.002$), as determined by paired Student's *t* test.

Table 2. Effect of ch225-LT on experimental lung metastases of human melanoma in athymic *nu/nu* mice depleted of asialo-GM1⁺ cells

Treatment ^a	No. of foci ^b						
PBS	>500	>500	>500	>500	>500	>500	>500
rhLT	>500	>500	>500	>500	>500	>500	>500
ch225-LT ^c	0	0	0	0	0	34	
ch225-LT plus anti-asialo-GM1 ^d	68	100	>500	>500	>500	>500	>500

^a Treatment was started 24 h after inoculation of tumor cells and consisted of i.v. injection of either PBS (0.1 ml), rhLT (0.5 μ g), or ch225-LT (16 μ g), daily over 7 consecutive days, as indicated.

^b Experimental lung metastases were induced by intravenous injection of 1×10^6 M24met melanoma cells into groups of six athymic *nu/nu* mice.

^c Differences in the number of metastatic foci between the groups of mice receiving the fusion protein, either depleted or not depleted of asialo-GM1⁺ cells, were statistically significant ($p \geq 0.002$), as determined by paired Student's *t* test.

^d Mice were depleted of asialo-GM1⁺ cells by injection of anti-asialo-GM1 antiserum (100 μ l) before and after tumor cell inoculation on days -3, 0, 7, 14, and 21.

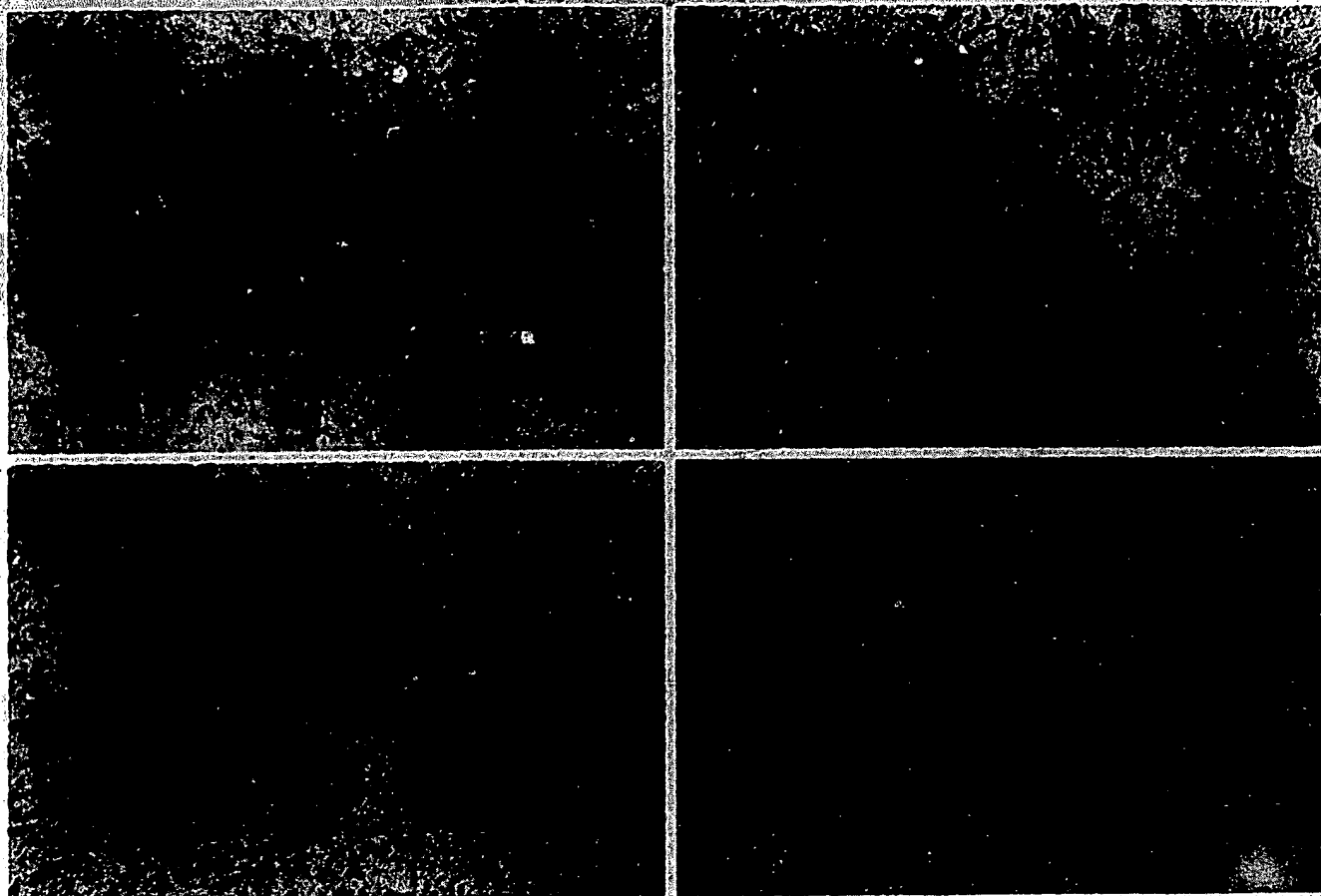


Fig. 3. Immunohistological characterization of inflammatory infiltrates induced by ch225-LT fusion protein in M24met tumors. Athymic *nu/nu* mice were injected s.c. with 2.5×10^6 M24met cells. After 10 days, four animals each were injected i.v. with $16 \mu\text{g}$ ch225-LT/day for 7 days. On day 7, after the start of therapy, sections of these tumors were prepared and subjected to anti-CD45 (A), Mac-1 (B), anti-asialo-GM1 (C), and anti-B220 (D) followed by immunostaining and counterstaining with hematoxylin. $\times 200$.

metastases to eradicate them in athymic *nu/nu* mice, but not in C.B17-*scid/scid* or *scid/beige* mice. A complex immunological mechanism appears to be involved in this antitumor effect that functions in the absence of mature T cells, but requires B, NK, and other asialo-GM1+ cells.

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